

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Feussner et al.

Art Unit: Unassigned

Application No. Unassigned
(U.S. National Phase of PCT/EP00/06539)

Examiner: Unassigned

Filed: January 8, 2002

For: 11-ARACHIDONATE-LIPOXYGENASE
MUTANTS

**AMENDMENTS TO SPECIFICATION, CLAIMS, AND ABSTRACT
MADE VIA PRELIMINARY AMENDMENT**

Amendments to the paragraph beginning at page 1, line 6:

The present invention relates to a method for producing a plant lipoxygenase with modified positional specificity and to the lipoxygenase obtained by said method and to the use thereof for the hydroperoxylation of arachidonic acid [to] at carbon atom 11.

Amendments to the paragraph beginning at page 2, line 28:

This problem is solved according to the invention by a method in which at least one amino acid is [ex]changed in a wild type LOX, preferably of potato tuber.

Amendments to the paragraph beginning at page 3, line 5:

Figure 3 shows the sequence of wild type LOX of potato tubers [SEQ ID NO:3]. The mutagenized amino acid position is underlined. Primers 1 [und] and 2 [SEQ ID NOS: 1 and 2, respectively] as used are also shown.

Amendments to the paragraph beginning at page 3, line 8:

In a preferred embodiment, the amino acids are [ex]changed in the region of the amino acid position 570 to 581 of potato tuber LOX. The above-indicated amino acid positions refer to the sequence under the access number S73865 in the EMBL data base or the sequence according to Fig. 3. The positions in LOXs of other plant species, which correspond to the amino acid positions 593 to 602 of *Cucumis sativus* lipoxygenase, can easily be determined

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by sequence comparisons between sequence X92890 and the further protein sequences, e.g., of soybean, potato, arabidopsis, tobacco or barley. The following Table 1 shows the result of an amino acid comparison between the cucumber-derived enzyme and the corresponding positions in the enzymes of other plants. The first group (15-LOX) shows a comparison between LOXs which at position 15 introduce a hydroperoxy group into an arachidonic acid molecule, while the second group (5-LOX) shows a comparison between sequences which introduce a hydroperoxy group at position 5.

Amendments to the paragraph beginning at page 5, line 1:

The present invention further relates to LOX mutants which are obtainable according to the above-described methods. The LOXs according to the invention can be produced with the help of the methods known from the prior art, for example directed mutagenesis, and subsequent protein expression. In particular mutants which after incubation with arachidonic acid yield at least 40%, preferably 50%, of the derivative perhydroxylated [to] at position 11 are considered to be inventive.

Amendments to the paragraph beginning at page 5, line 27:

Finally, new plants or plant parts can be regenerated from the above-mentioned cells by *in vitro* culturing methods. For the production of such transgenic plants the known transformation system can [e.g.] be used, e.g., on the basis of [a] *Agrobacteria* and Ti plasmid derivatives.

Amendments to the paragraph beginning at page 6, line 4:

Particularly preferred is an arachidonic acid derivative which contains a hydroperoxy group at position 11. The derivative can then easily be converted into the hydroxy derivative. The thus available 11S-HPETE can be used for producing the alcohols, aldehydes and dicarboxylic acids shown below. The enzyme hydroperoxide lyase is [e.g.] contained in extracts of cucumber seedlings, for example. 2E- and 3Z-nonenal and their alcohols are important flavorings in foodstuff (e.g., cucumbers).

Amendments to the paragraph beginning at page 7, line 15:

For bacterial expression of wild type LOX and LOX mutant and for directed mutagenesis, use was made of the plasmid pet3b (Novagen, Germany) which contained the cDNA of the

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potato tuber LOX as insert (pET-LOX1; cf. Geerts, A., Feltkamp, D., Rosahl, S. (1994) Expression of lipoxygenase in wounded tubers of *solanum tuberosum* L., *Plant Physiol.* 105: 269-277). Mutagenesis was carried out by using the QuikChange Mutagenesis Kit from Stratagene (Heidelberg, Germany). Oligonucleotides containing the appropriate base exchanges were purchased from MWG-Biotech (Ebersberg, Germany). To analyze the mutation, an additional conservative base exchange was introduced to construct a new restriction cleavage site. In addition, the mutation was sequenced and at least five different bacterial clones were expressed and used for analyzing the enzymatic characteristics. Expression of pET-LOX1 [und] and its mutant was performed as described by Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) *FEBS Lett.* 431, 433-436. Cells from 1 liter cultures were resuspended in 5-7 ml lysis buffer and disrupted by using a sonifier tip with pulses each of 30 seconds, and cellular debris was pelleted.

Amendments to the paragraph beginning at page 8, line 4:

For product analysis, 0.9 ml of cell lysates [was] were incubated with 0.9 mM arachidonic acid (final concentration) in 100 mM Tris buffer, pH 7.5, for 30 minutes at room temperature. Reaction was stopped by the addition of sodium borohydride to convert the hydroperoxy fatty acids formed to the corresponding hydroxy compounds. The samples were acidified to pH 3 and the lipids were extracted (cf. Bligh, E.G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* 37, 911-917). The lower chloroform phase was recovered and the solvent was evaporated. The remaining lipid was dissolved with 0.1 ml methanol, and aliquots were subjected to HPLC analysis.

Amendments to the paragraph beginning at page 8, line 15:

HPLC analysis was carried out on a Hewlett Packard 1100 HPLC system coupled to a diode detector. RP-HPLC of the free fatty acid derivatives was carried out on a Nucleosil C-18 column (Macherey-Nagel, 250 x 4 mm, 5 µm particle size) with a solvent system of methanol/water/acetic acid (85/15/0.1; v/v/v) and at a flow rate of 1 ml/min. Absorption at 234 nm (absorption of the conjugated diene system of the hydroxy fatty acids) and at 210 nm (polyenoic fatty acids) [were] was recorded accordingly. Straight-phase HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (HP, Waldbronn, Germany; 250 x 4.6 mm, 5 µm particle size) with a solvent system of n-hexane/2-propanol/acetic acid (100/2/0.1, v/v/v) at a flow rate of 1ml/min. The enantiomer